Virology 496 (2016) 215–218

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The green tea molecule EGCG inhibits Zika virus entry

Bruno M. Carneiro [a,*], Mariana N. Batista [a], Ana Cláudia S. Braga [a], Maurício L. Nogueira [b], Paula Rahal [a]

[a] Laboratory of Genomic Studies, Sao Paulo State University - UNESP, São José do Rio Preto, SP, Brazil
[b] Departamento de Doenças Infecciosas e Parasitárias, FAMERP, 15090-000 São José do Rio Preto, SP, Brazil

ARTICLE INFO

Article history:
Received 16 May 2016
Returned to author for revisions
14 June 2016
Accepted 15 June 2016
Available online 23 June 2016

Keywords:
Zika virus
(−)-Epigallocatechin gallate
Antiviral
Green-tea

ABSTRACT

During ZIKV the outbreak in Brazil it was observed an increase of almost 20 times the number of reported cases of microcephaly in newborn babies. There is no vaccine or approved drug available for the treatment and prevention of infections by this virus. EGCG, a polyphenol present in green tea has been shown to have an antiviral activity for many viruses. In view of the need for the development of a drug against a Brazilian strain of ZIKV, we assessed the effect of EGCG on ZIKV entry in Vero E6 cells. The drug was capable of inhibiting the virus entry by at least 1-log (> 90%) at higher concentrations (> 100 μM). The pre-treatment of cells with EGCG did not show any effect on virus attachment. This is the first study to demonstrate the effect of EGCG on ZIKV indicating that this drug might be possibility to be used for prevention of Zika virus infections.

1. Introduction

The Zika (ZIKV) virus, which causes the Zika fever, was first identified in 1947 in Uganda, in a forest with the same name (Dick et al., 1952). Since its initial identification, sporadic cases were reported in Southeast Asia and sub-Saharan Africa (Hayes, 2009). In 2007, the first outbreak caused by this virus was reported in Micronesia and more recently, in French Polynesia and Easter Island (Cao-Lormeau et al., 2014; Duffy et al., 2009). The first indigenous case of Zika virus in Brazil was confirmed in May of 2015 (Zanluca et al., 2015).

During the outbreak in Brazil it was observed an increase of almost 20 times the number of cases of microcephaly in reported newborn babies. Health officials believe there may be a link between the virus and this clinical condition. So far, no other *Flavivirus* was shown to have teratogenic capacity (Fauci and Morens, 2016). Nonetheless, two research groups have recently and independently demonstrated the presence of the virus in both brain tissue and amniotic fluid of aborted fetuses, strengthening the virus's link to cases of microcephaly (Calvet et al., 2016; Mlakar et al., 2016). Finally, Rasmussen et al. (2016) demonstrated a strong causal relationship between ZIKV and microcephaly and associated it to brain anomalies in fetuses and infants. Despite the efforts of many researchers, up to this date there is no vaccine available for the treatment and prevention of infections caused by this virus. Besides, no drug with antiviral activity against this virus has been authorized. Such measures could greatly reduce cases of neurological damage in fetuses.

The (−)-epigallocatechin gallate (EGCG) (Fig. 1), a polyphenol present in large quantities in green tea has been shown to have an intense antiviral activity for many viruses, including the human immunodeficiency virus (HIV), herpes simplex virus (HSV), influenza virus (FLU) and hepatitis C virus (HCV) (Calland et al., 2012; Isaacs et al., 2008; Nance et al., 2009; Song et al., 2005). These studies demonstrated that EGCG mainly acts by inhibiting the entry of the virus into the host cell. Furthermore, it has been shown that the administration of this drug is safe for healthy individuals (Chow et al., 2003). Considering the fact that EGCG has an antiviral activity against different viruses including HCV, a virus belonging to the same family of ZIKV, and taking in account the need for the development of a drug against ZIKV, we assessed the effect of EGCG on ZIKV entry in Vero E6 cells.

2. Methods

2.1. Virus preparation

It was used a strain of Zika virus ($ZIKV^{BR}$) isolated from a febrile case in the state of Paraíba (Faria et al., 2016), northeastern Brazil, courtesy of Dr. Pedro Vasconcelos, Instituto Evandro Chagas, Brazil. A sample of this virus was inoculated in *Aedes albopictus* cells clone C6/36 and incubated for 10–12 days until onset of cytopathic effects. Subsequently a freeze-thaw cycle was done, the

* Corresponding author.
E-mail address: brunocopo@yahoo.com.br (B.M. Carneiro).

http://dx.doi.org/10.1016/j.virol.2016.06.012
0042-6822/

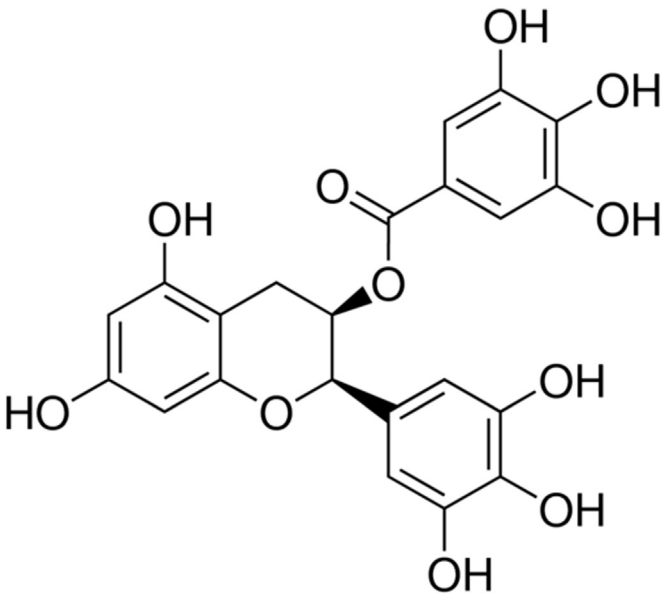

Fig. 1. Structural formula of (−)-epigallocatechin gallate (EGCG).

supernatant was clarified, aliquoted and stored at −80 °C until it was again necessary. The virus was received at its second passage after sample collection and all experiments were done with a third passage virus. An African Strain (MR766) was used to confirm the results obtained with the Brazilian strain. The virus sample was gently donated by Dr. Amilcar Tanuri, Universidade Federal do Rio de Janeiro, Brazil. Upon the receipt of the sample it was inoculated on Vero E6 cells and it was performed the same procedure used for the other strain.

2.2. Virucidal effect of EGCG

In order to determine the anti-ZIKV effect of EGCG, Vero E6 cells were used for evaluation. Briefly, Vero E6 cells (1.5×10^5) were seeded in each well of a 12-well plate and incubated at 37° C with 5% CO_2 for 24 h prior to infection. Approximately 10^6 focus forming units of $ZIKV^{BR}$ or MR766 were mixed to different concentrations of pure EGCG (Sigma Aldrich, >95% purity) and incubated for 1 h at room temperature to assess the virucidal activity of this compound. Following, the drug-treated viral supernatant were serially diluted in Dulbecco modified essential medium (DMEM) and added to the plates. After incubation for 1 h at 37° C for viral adsorption, 1 mL of DMEM supplemented with 2% fetal bovine serum (FBS) (Cultilab) and 1% carboxymethylcellulose sodium salt (Sigma-aldrich) were added to the wells and plate was incubated for 96 h. After four days media was removed, cells were fixed with 10% formaldehyde and stained with 1% crystal violet (Sigma-aldrich) diluted in 20% ethanol. Foci were counted and compared to control (diluent only).

2.3. Cytotoxicity of EGCG on Vero E6 cells

In order to confirm that the inhibitory effect observed was not a result of a change in cellular conditions, we assessed the cytotoxicity of EGCG in Vero E6 cells. For this assay, 5×10^3 Vero E6 cells were added to each well of a 96-well plate and incubated for 24 h at 37° C. Media was removed and replaced with DMEM containing different concentrations of EGCG (0–200 μM). The effects of the EGCG on the cells were determined at 1, 24, 48 and 72 h post addition of the drug to culture media. The supernatants were removed, and a solution of 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT −1 μg/mL) was added to each well and the plate was incubated for 30 min at 37 °C. Subsequently to incubation, the MTT crystals were solubilized with 100 μL of Dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm.

2.4. Pre-treatment of Vero E6 cells with EGCG

We also tested a pre-treatment of cells with EGCG before performing the infection. For this, 24 h before the assay Vero E6 cells were added in a 12-well plate and incubated at 37° C. In the following day, the culture growth medium was replaced with DMEM containing 50 μM EGCG and incubation proceeded for 1 h at 37° C. After this period, the medium containing the drug was removed and the cell monolayer was washed 3 times with PBS to eliminate any trace of EGCG. Then the virus was inoculated on the treated cells and the remainder of the procedure was similar to that used in the test of virucidal activity.

3. Results and discussion

Despite its identification in 1947 (DICK et al., 1952), the Zika virus (ZIKV) began to draw world attention only in recent years, mainly because of its possible association with cases of microcephaly and neuronal problems in fetuses (Rasmussen et al., 2016). Several efforts are being made in the search for a vaccine or an effective drug that can inhibit or reduce the harmful effects caused by this virus. EGCG is a natural drug with known antiviral effect and in this study we evaluated their activity against ZIKV, mainly it's the interference with the virus entry process in a host cell.

We first evaluated the virucidal ability of EGCG by incubating the $ZIKV^{BR}$ virus with different concentrations of this drug. In this assay we observed a reduction of at least 1-log (>90%) in the number of observed foci when the virus was pre-incubated for 1 h with 200 μM of EGCG, indicating that this compound has potent virucidal activity against the ZIKV (Fig. 2(A)). In lower concentrations, the drug had a lesser ability to inhibit virus entry and at the lowest (5 μM) concentration evaluated, we observed a reduction of only 5% in foci number. By non-linear regression we calculated the EC_{50} of this compound to be 21.4 μM. Similar results were observed when EGCG was tested against the MR766 strain (Fig. 2(B)). However, this virus showed to be more sensible than the Brazilian strain. At the 25 μM concentration, EGCG inhibited 85% of MR766 strain entry, whereas it was necessary twice (50 μM) this concentration to obtain the same inhibition of the $ZIKV^{BR}$.

These results suggest that the compound exerts a direct action on viral particle, inhibiting its binding to the target cell. A similar result was observed by other researchers when they tested this drug against HCV, but not to other viruses of the *Flaviviridae* family, since the yellow fever virus (YFV), bovine diarrhea (BVDV) and Sindbis virus (SINV) were not inhibited by EGCG (Calland et al., 2012). Besides HCV, EGCG has also demonstrated activity against HIV entry in host cells and in this case it was established that the virucidal effect of the compound relates to its ability to destroy the envelope phospholipids by destabilizing the viral particle and thus leading to the destruction of the virus (Yamaguchi et al., 2002). We believe this may also be the mechanism by which the drug inhibited ZIKV in our tests, however, more studies are needed to confirm this hypothesis. Regarding the difference in sensitivity observed between both strains to the EGCG treatment, when the amino acids sequences of envelope protein were compared, we observed several changes, being the modification on protein composition what could explain the difference on the drug effectiveness.

Despite being a natural compound, some studies have shown that EGCG at higher concentrations can be toxic to various cell

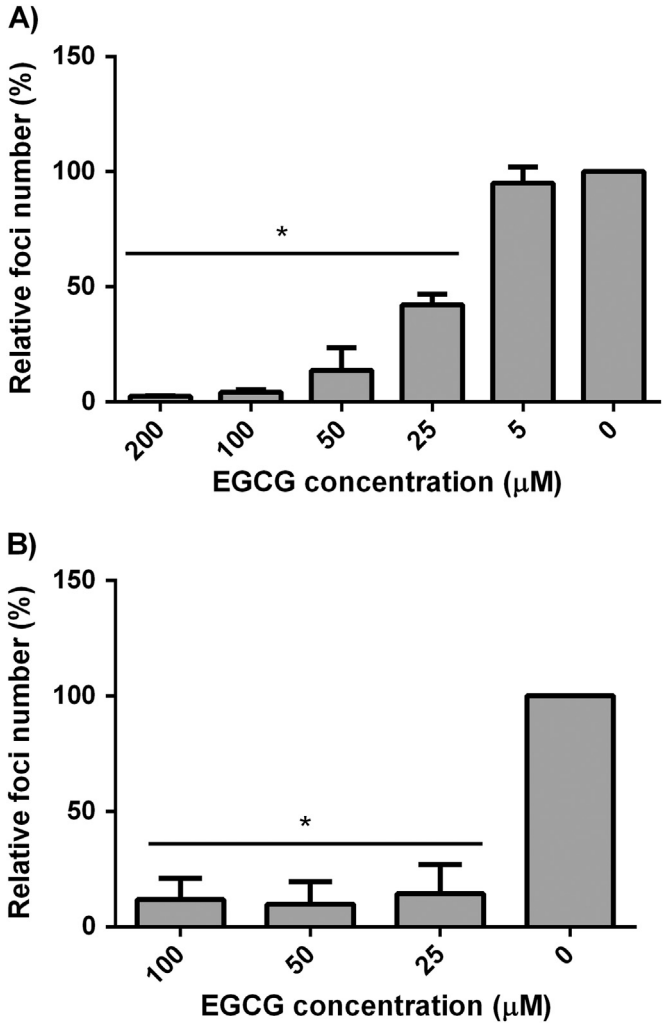

Fig. 2. Inhibition of ZIKV entry by EGCG. Approximately 10^6 FFU of ZIKV^{BR} (A) or MR766 (B) were incubated with different concentrations of EGCG for 1 h at room temperature. Then the supernatant was serially diluted and used to infect Vero E6 cells in 12-well plates. After 96 h the cells were fixed with 10% formaldehyde and stained with 1% crystal violet. The results shown are means (± SD) of two independent events, expressed as relative values compared to the untreated control. * $P < 0.05$ vs. control.

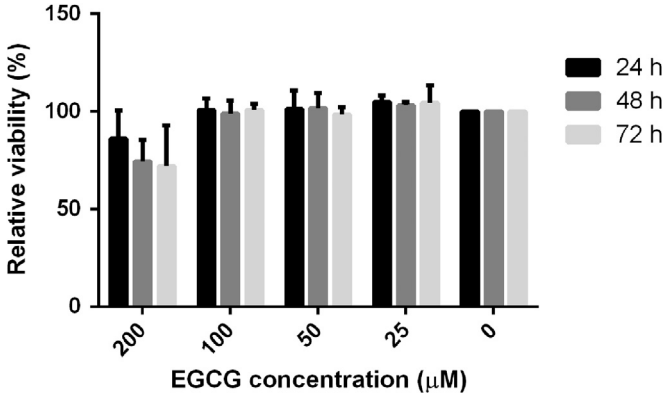

Fig. 3. Viability of EGCG treated Vero E6 cells. Cells were cultured in the presence of different concentrations of EGCG for 24, 48 and 72 h. After treatment time, EGCG-containing medium was removed and MTT solution (1 mg/mL) was added to the cells and incubated for 1 h. Formazan crystals were solubilized with 100 μL of DMSO and OD was determined at 570 nm. Results are means (± SD) of 4 replicates from three independent experiments and are expressed as relative values compared to untreated cells.

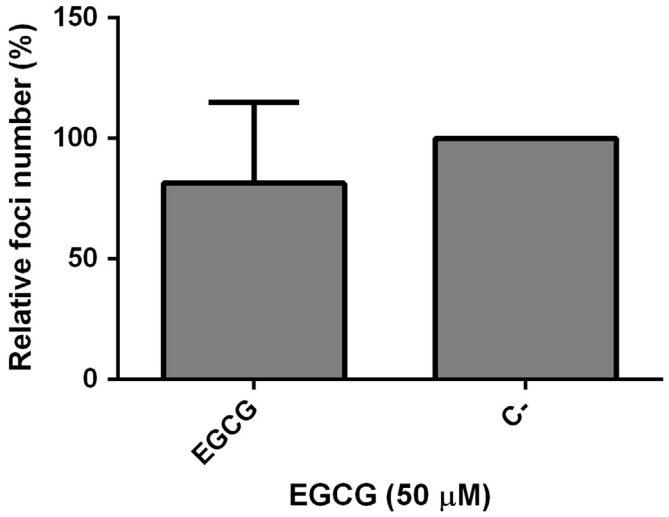

Fig. 4. Effect of EGCG on cellular receptors. Vero E6 were pre-incubated with 50 μM of EGCG for 1 h, EGCG-containing culture medium was removed and cells extensively washed with PBS. Cells were then challenged with ZIKV inoculum and incubated for 96 h. After incubation time, cells were fixed with 10% formaldehyde and stained with 1% crystal violet. Results are means (± SD) from two independent experiments and are expressed as relative values compared to untreated cells.

lines (Weisburg et al., 2004). Among all drug concentrations that were evaluated, only 200 μM showed some toxicity, reducing cell viability by 20% after 72 h incubation (Fig. 3). At the concentration of 100 μM we observed a small decrease of $< 5\%$ also after 72 h. Concentrations above 300 μM led to a reduction of more than 50% cell viability (data not shown). Therefore, with these results we confirmed that the reduction of infectivity ZIKV EGCG was not due to an interference of the cell conditions, but due to the direct activity on the viral particle.

In addition to their virucidal capacity, some studies have shown that EGCG can act by blocking necessary receptors for viral attachment to the target cell, as in the case of HIV where drug binds to CD4 expressed on T cells and inhibit the virus binding to this site, blocking viral infection (Williamson et al., 2006). In the pretreatment assay we did not observe any influence of the drug on viral infection at the concentration tested, suggesting that EGCG has no action on the expression or in blocking the cellular receptors used by ZIKV during the entry process in the host cell (Fig. 4). Although present for HIV, this antiviral mechanism was not observed for any other virus susceptible to EGCG (Steinmann et al., 2013). Ciesek et al. (2011) actively looked for this mechanism in Huh7.5 cells and did not observe any influence of this drug on the expression of cell receptors necessary for HCV to entry on those cells.

In conclusion, we have demonstrated in this study that EGCG, a natural compound found in abundance in many foods, especially in green tea, inhibits *in vitro* the entry of ZIKV into the host cell. The mechanism by which this inhibition occurs is probably related to the direct interaction of the drug with lipid envelope, leading to a subsequent destruction of the virus particle. We also presented that the action of this drug is not related to blocking or reducing the expression of cell receptors used by the virus in their entry process. Despite these encouraging results, some considerations regarding this compound pharmacokinetics must be done. Previous studies have demonstrated that EGCG is chemically unstable; it has a low permeability membrane as well as being rapidly metabolized by the organism. The maximum plasma concentration reached by this drug is approximately 7 μM (Steinmann et al., 2013), almost 15 times smaller than the optimal concentration for the elimination of ZIKV. However, some studies have shown that the bioavailability of EGCG can be considerably

increased by chemical modifications such as peracetylation (Lambert et al., 2006) or by using nanoencapsulation (Fangueiro et al., 2016).

Another point to be considered is that in a study with rats, EGCG has shown that it can easily cross the placental barrier and spread to many fetal tissues, especially the brain, eyes and heart (Chu et al., 2007). Considering the possible teratogenic capacity of ZIKV, this feature of EGCG appears to be quite interesting since even though it does not completely eliminate the virus from fetal tissue, the drug can mitigate the possible damage to the embryo caused by the virus. Preliminary studies in rats also showed that even after maternal intake of high concentrations of EGCG, it wasn't observed any adverse effect on the formation of embryos, even during the period of organogenesis (Isbrucker et al., 2006). These studies therefore suggest that the drug appears to be safe for consumption by pregnant women and can be an important tool to help prevent damage to human embryos. Yet, more studies are necessary.

Finally, this is the first report demonstrating that EGCG is a good possibility to be used in therapy and prevention of infections caused by the Zika virus. Nevertheless, before its effective implementation, factors such as their bioavailability and the safety evaluation of its use for pregnant women's should be improved.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP), Brazil. Grant numbers 2013/21719-3, 2014/22199-6 and 2014/22198-0.

Acknowledgements

The authors are grateful to Dr. Pedro Vasconcelos (Instituto Evandro Chagas, Brazil) and Dr. Amilcar Tanuri (Universidade Federal do Rio de Janeiro, RJ, Brazil) for providing the virus isolates used in this study. The authors would also like to thank all members of the Laboratory of Genomics Studies (IBILCE-UNESP, BR) for their helpful suggestions and discussions.

References

Calland, N., Albecka, A., Belouzard, S., Wychowski, C., Duverlie, G., Descamps, V., Hober, D., Dubuisson, J., Rouillé, Y., Séron, K., 2012. (-)-Epigallocatechin-3-gallate is a new inhibitor of hepatitis C virus entry. Hepatology 55, 720–729. http://dx.doi.org/10.1002/hep.24803.

Calvet, G., Aguiar, R.S., Melo, A.S.O., Sampaio, S.A., de Filippis, I., Fabri, A., Araujo, E.S. M., de Sequeira, P.C., de Mendonça, M.C.L., de Oliveira, L., Tschoeke, D.A., Schrago, C.G., Thompson, F.L., Brasil, P., dos Santos, F.B., Nogueira, R.M.R., Tanuri, A., de Filippis, A.M.B., 2016. Detection and sequencing of Zika virus from amniotic fluid of fetuses with microcephaly in Brazil: a case study. Lancet Infect. Dis. . http://dx.doi.org/10.1016/S1473-3099(16)00095-5

Cao-Lormeau, V.-M., Roche, C., Teissier, A., Robin, E., Berry, A.-L., Mallet, H.-P., Sall, A.A., Musso, D., 2014. Zika virus, French polynesia, South pacific, 2013. Emerg. Infect. Dis. 20, 1085–1086. http://dx.doi.org/10.3201/eid2006.140138.

Chow, H.-H.S., Cai, Y., Hakim, I.A., Crowell, J.A., Shahi, F., Brooks, C.A., Dorr, R.T., Hara, Y., Alberts, D.S., 2003. Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. Clin. Cancer Res. 9, 3312–3319.

Chu, K.O., Wang, C.C., Chu, C.Y., Choy, K.W., Pang, C.P., Rogers, M.S., 2007. Uptake and distribution of catechins in fetal organs following in utero exposure in rats. Hum. Reprod. 22, 280–287. http://dx.doi.org/10.1093/humrep/del353.

Ciesek, S., von Hahn, T., Colpitts, C.C., Schang, L.M., Friesland, M., Steinmann, J., Manns, M.P., Ott, M., Wedemeyer, H., Meuleman, P., Pietschmann, T., Steinmann, E., 2011. The green tea polyphenol, epigallocatechin-3-gallate, inhibits hepatitis C virus entry. Hepatology 54, 1947–1955. http://dx.doi.org/10.1002/hep.24610.

Dick, G.W.A., Kitchen, S.F., Haddow, A.J., 1952. Zika virus. I. Isolations and serological specificity. Trans. R. Soc. Trop. Med. Hyg. 46, 509–520.

Duffy, M.R., Chen, T.-H., Hancock, W.T., Powers, A.M., Kool, J.L., Lanciotti, R.S., Pretrick, M., Marfel, M., Holzbauer, S., Dubray, C., Guillaumot, L., Griggs, A., Bel, M., Lambert, A.J., Laven, J., Kosoy, O., Panella, A., Biggerstaff, B.J., Fischer, M., Hayes, E.B., 2009. Zika virus outbreak on Yap Island, Federated States of Micronesia. N. Engl. J. Med. 360, 2536–2543. http://dx.doi.org/10.1056/NEJMoa0805715.

Fangueiro, J.F., Calpena, A.C., Clares, B., Andreani, T., Egea, M.A., Veiga, F.J., Garcia, M. L., Silva, A.M., Souto, E.B., 2016. Biopharmaceutical evaluation of epigallocatechin gallate-loaded cationic lipid nanoparticles (EGCG-LNs): in vivo, in vitro and ex vivo studies. Int. J. Pharm. 502, 161–169. http://dx.doi.org/10.1016/j.ijpharm.2016.02.039.

Faria, N.R., Azevedo, R., do, S., da, S., Kraemer, M.U.G., Souza, R., Cunha, M.S., Hill, S. C., Thézé, J., Bonsall, M.B., Bowden, T.A., Rissanen, I., Rocco, I.M., Nogueira, J.S., Maeda, A.Y., Vasami, F.G., da, S., Macedo, F.L., de, L., Suzuki, A., Rodrigues, S.G., Cruz, A.C.R., Nunes, B.T., Medeiros, D.B., de, A., Rodrigues, D.S.G., Nunes Queiroz, A.L., Silva, E.V.P., da, Henriques, D.F., Travassos da Rosa, E.S., de Oliveira, C.S., Martins, L.C., Vasconcelos, H.B., Casseb, L.M.N., Simith, D., de, B., Messina, J.P., Abade, L., Lourenço, J., Alcantara, L.C.J., Lima, M.M., de, Giovanetti, M., Hay, S.I., de Oliveira, R.S., Lemos, P., da, S., Oliveira, L.F., de, de Lima, C.P.S., da Silva, S.P., Vasconcelos, J.M., de, Franco, L., Cardoso, J.F., Vianez-Júnior, J.L., da, S.G., Mir, D., Bello, G., Delatorre, E., Khan, K., Creatore, M., Coelho, G.E., de Oliveira, W.K., Tesh, R., Pybus, O.G., Nunes, M.R.T., Vasconcelos, P.F.C., 2016. Zika virus in the Americas: early epidemiological and genetic findings. Science 352, 345–349. http://dx.doi.org/10.1126/science.aaf5036.

Fauci, A.S., Morens, D.M., 2016. Zika virus in the Americas – yet another arbovirus threat. N. Engl. J. Med. 374, 601–604. http://dx.doi.org/10.1056/NEJMp1600297.

Hayes, E.B., 2009. Zika virus outside Africa. Emerg. Infect. Dis. 15, 1347–1350. http://dx.doi.org/10.3201/eid1509.090442.

Isaacs, C.E., Wen, G.Y., Xu, W., Jia, J.H., Rohan, L., Corbo, C., Di Maggio, V., Jenkins, E. C., Hillier, S., 2008. Epigallocatechin gallate inactivates clinical isolates of herpes simplex virus. Antimicrob. Agents Chemother. 52, 962–970. http://dx.doi.org/10.1128/AAC.00825-07.

Isbrucker, R.A., Edwards, J.A., Wolz, E., Davidovich, A., Bausch, J., 2006. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 3: teratogenicity and reproductive toxicity studies in rats. Food Chem. Toxicol. 44, 651–661. http://dx.doi.org/10.1016/j.fct.2005.11.002.

Lambert, J.D., Sang, S., Hong, J., Kwon, S.-J., Lee, M.-J., Ho, C.-T., Yang, C.S., 2006. Peracetylation as a means of enhancing in vitro bioactivity and bioavailability of epigallocatechin-3-gallate. Drug Metab. Dispos. 34, 2111–2116. http://dx.doi.org/10.1124/dmd.106.011460.

Mlakar, J., Korva, M., Tul, N., Popović, M., Poljšak-Prijatelj, M., Mraz, J., Kolenc, M., Resman Rus, K., Vesnaver Vipotnik, T., Fabjan Vodušek, V., Vizjak, A., Pižem, J., Petrovec, M., Avšič Županc, T., 2016. Zika virus associated with microcephaly. N. Engl. J. Med. 374, 951–958. http://dx.doi.org/10.1056/NEJMoa1600651.

Nance, C.L., Siwak, E.B., Shearer, W.T., 2009. Preclinical development of the green tea catechin, epigallocatechin gallate, as an HIV-1 therapy. J. Allergy Clin. Immunol. 123, 459–465. http://dx.doi.org/10.1016/j.jaci.2008.12.024.

Rasmussen, S.A., Jamieson, D.J., Honein, M.A., Petersen, L.R., 2016. Zika virus and birth defects—reviewing the evidence for causality. N. Engl. J. Med. . http://dx.doi.org/10.1056/NEJMsr1604338

Song, J.-M., Lee, K.-H., Seong, B.-L., 2005. Antiviral effect of catechins in green tea on influenza virus. Antivir. Res. 68, 66–74. http://dx.doi.org/10.1016/j.antiviral.2005.06.010.

Steinmann, J., Buer, J., Pietschmann, T., Steinmann, E., 2013. Anti-infective properties of epigallocatechin-3-gallate (EGCG), a component of green tea. Br. J. Pharmacol. 168, 1059–1073. http://dx.doi.org/10.1111/bph.12009.

Weisburg, J.H., Weissman, D.B., Sedaghat, T., Babich, H., 2004. In vitro cytotoxicity of epigallocatechin gallate and tea extracts to cancerous and normal cells from the human oral cavity. Basic Clin. Pharmacol. Toxicol. 95, 191–200. http://dx.doi.org/10.1111/j.1742-7843.2004.pto_950407.x.

Williamson, M.P., McCormick, T.G., Nance, C.L., Shearer, W.T., 2006. Epigallocatechin gallate, the main polyphenol in green tea, binds to the T-cell receptor, CD4: potential for HIV-1 therapy. J. Allergy Clin. Immunol. 118, 1369–1374. http://dx.doi.org/10.1016/j.jaci.2006.08.016.

Yamaguchi, K., Honda, M., Ikigai, H., Hara, Y., Shimamura, T., 2002. Inhibitory effects of (−)-epigallocatechin gallate on the life cycle of human immunodeficiency virus type 1 (HIV-1). Antivir. Res. 53, 19–34.

Zanluca, C., de Melo, V.C.A., Mosimann, A.L.P., Dos Santos, G.I.V., Dos Santos, C.N.D., Luz, K., 2015. First report of autochthonous transmission of Zika virus in Brazil. Mem. Inst. Oswaldo Cruz 110, 569–572. http://dx.doi.org/10.1590/0074-02760150192.